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Comparative Evaluation of Developed Carrier Based Bioformulations Bearing Multifarious PGP Properties and Their Effect on Shelf Life under Different Storage Conditions

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ABSTRACT

The current study was undertaken to determine the bio-formulations of two economically viable carriers (talc and sugarcane bagasse) based preparations by using plant growth promoting (PGP) strains. A total of 20 bacterial isolates were recovered from rice agricultural fields of Alibag and Chiplun, Maharashtra, India. Recovered bacterial isolates were screened on the basis of PGP properties like phosphate, zinc solubilization, siderophore, indole acetic acid and ammonia production. On the basis of plant growth promoting potential, two bacterial isolates i.e. A2 and CP19 were selected and showed 98% homology with *Providencia vermicola* and *Klebsiella pneumoniae* based on 16S rDNA sequence analysis. The selected PGP strains were co-inoculated in combination with each other after finding the compatibility of both strains and designated as consortium Cl. The developed consortium was utilized for bio-formulation preparation with talc and sugarcane bagasse carrier, respectively. The viable cell count of both bio-formulations was determined at the end of 2^{nd}, 4^{th}, 11^{th},18^{th}, 25^{th}, 40^{th}, 55^{th} and 70^{th} day of incubation at different storage conditions (at 4^0C, room temperature and outdoor conditions). The comparative assessment of shelf life revealed that the viability of bacterial consortia Cl was found maximum in talc based bioformulations as compared to bagasse bioformulation after 70 days of storage (DAS). The consortia based talc bioformulation was found to confer a significant increase in the shelf life parameters under different conditions. The results of the present study suggest that talc based bioformulation of bacterial consortia of *Providencia vermicola* A2 and *Klebsiella pneumoniae* CP19 can be used as an effective carrier for easy application, storage and can be applied at the site of action for economizing production process and longer shelf life.

Keywords PGPR, Bioformulation, Siderophore, Talc, Bagasse.

INTRODUCTION

Microbial diversity are chief components of the soil system which interconnects with plants in diverse capacities and among them, interactions in the rhizospheric region is of the prime significance to the plant (Upadhayay et al. 2018). These valuable interactions in the rhizosphere are known to affect plant growth

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and development. Rhizobacteria are implicated either as biofertilizers or biopesticides which play an imperative role in our pursuit for evergreen agriculture by ameliorating twin provisions of soil fertility and crop productivity (Singh and Singh 2017). Moreover, the role of rhizobacteria and other soil associated microorganisms (such as Mycorrhizal fungi) has been depicted in crop biofortification through enhanced acquisition of micronutrients from soil to edible portions of plants (Upadhayay et al. 2018, 2019a, 2019b). At the outset, rhizobacteria perk up the fertility of soil by triumphing over the inadequacies integrated with the undue utilization of chemical fertilizers, viz., soil health deterioration, rising expenditures (both input and output) and correlated health hazards for living organisms including mankind (Singh and Prasad 2014, Parveen et al. 2018, Kaviya et al. 2019). This information mounts the perception of integrated nutrient management system for sustainable agriculture so that crop productivity is improved in an efficient and eco-friendly manner (Dubey et al. 2014).

However, the biggest downside in attaining agricultural sustainability has been the malfunctioning of beneficial rhizospheric microorganisms, such as efficiently commercialization of PGPR due to a host of factors, viz. environmental, climatic and species-specific or niche-specific tripartite soil-plant-microbe interactions (Maheshwari et al. 2015). The additional facet which has decreased the estimated industrial recognition of utilizing PGPR as biofertilizers is a lack of appropriate and economically viable carrier. The advantageous effect of these bacterial populations can be optimally deliberated only when carrier based formulations, are acquiescent for field level appliances and made accessible in the customers. Beneficial bacteria are currently applied in both forms of solid and liquid carrier based inoculants preparations in a range of agro economically vital plants (Dutta and Thakur 2017). However, the triumph of carrier based formulations primarily relies on the bacterial strain used for inoculant production. Liquid formulations possess their own advantages for smaller scale agricultural systems, such as easier to handle, working faster, being uniform and store; which solid based formulations are inexpensive and easier to manufacture. Historically these carriers have been classified as inert materials (alginate, perlite, talcum powder, vermiculite and other inorganic minerals), soils (coal, clays and peat) and plant waste materials (compost, farm yard manure and soybean meal) (Klayraung et al. 2009).

However, commercial inoculants production involved the employment of different plant, soil or inert material based carriers with immense surfeit of soil bacteria irrespective of genera or species but focused primarily on the purposeful traits (zinc solubilizers, siderophore production, nitrogen fixers that can be diazotrophic or symbiotic and biocontrol agents). These biofertilizers have mostly been developed on single species theory considering their respective functional attributes; however, two diverse bacterial genera in economical carriers as consortia having different PGP characteristics are yet to be investigated for their valuable interconnections with plants. Thus,.in this study, a formulation including consortia of beneficial bacterial strains (or species) with an easy-to-use and economical carrier material has been studied for proficient deliverance of bioinoculants to the particular crop. In this work, two promising strains *Providencia vermicola* A2 and *Klebsiella pneumoniae* CP19 with multiple PGP characters were selected and analyzed with two economically viable carriers (talc and bagasse) for study. Both the bacteria (A2 and CP19) were studied in combination to each other as two-species bacterial consortium. To measure the effectiveness of the economical carriers and consortia theory, an *in vitro* study was carried out to evaluate the effect on shelf life parameters of co-inoculant bio-formulation under various storage conditions.

MATERIALS AND METHODS

Soil sample collection and bacterial isolation

Soil samples were collected from the rice agriculture fields of Alibag and Chiplun, Maharashtra, India. This site is situated at an altitude of 243.84 m above mean sea level, 29^0N latitude and 79.3^0 E longitudes; 1g soil sample was serially diluted up to 10^6 dilutions and plated in nutrient agar; 200 µL of diluted sample was poured in 25 mL of nutrient agar medium plate. After homogenizing properly, inoculated plates were incubated at 28 ± 2^0C for 24 h 20 bacterial colonies were selected based on their morphological characteristics.

Screening of bacterial isolates with plant growth promoting properties

All the recovered bacterial cultures were screened qualitatively and quantitatively for siderophore production, zinc solubilization and production of indole acetic acid and ammonia.

Zinc solubilization

Screening of Zn solubilizing bacteria (ZSB) was done on basal agar medium supplemented with 0.1% ZnO according to Saravanan et al. (2003). Recovered bacteria were examined for their Zn solubilizing potential based on halo zone formation around the bacterial colonies.

Phosphate solubilization

Bacterial cultures were spot inoculated on sterile Pikovskaya agar medium (HI media) and incubated for 4 to 6 days at 28^0C. Formation of halo zone around bacterial colony signifies phosphate solubilization by the bacteria (Pikovskaya 1948).

Siderophore production

Siderophore production in selected bacterial isolates was detected by using universal method of Schwyn and Neilands (1987). Bacterial cultures were spot inoculated on sterile CAS agar and incubated at 28 ± 2^0C for 60 to 72 h. Formation of orange or yellow halo zone around the bacterial colony indicates a positive result for siderophore production. CAS agar plates were prepared by mixing Chrome Azurol'S (60.5 mg) in distilled H_2O (50 mL), to which 10 mg 1 mM $FeCl_3$. $6H_2O$ in 10 mM HCL was added gradually. The mixture was then added to HDTMA solution (72.9 mg in 40 mL distilled H_2O). The obtained dark blue color solution was autoclaved at 15 Ib psi for 20 min. Sterilized nutrient agar (300 mL) was mixed with CAS solution in the ratio of 1 : 10 (CAS solution : Media).

Indole acetic acid production

Actively grown bacterial culture was inoculated in 5 mL luria broth, supplemented with 100 µg/ mL tryptophan. After incubation at 28 ± 2^0C for 48 h, broth was centrifuged for 10 min at 10,000 rpm. After centrifugation, one ml culture supernatant was mixed with Salkovaski reagent (2 mL) and incubated at 30^0C for 25 min in dark to observe color change. Development of pink color indicates a positive test for IAA production. Absorbance of the colored mixture was monitored at 530 nm by using UV/visible spectrophotometer (Patten and Glick 2002).

Ammonia production

Actively grown bacterial cultures were inoculated in 10 mL peptone water and incubated for 72 h at 28 ± 2^0C in a rotatory shaker at 100 rpm. Ammonia production was examined by supplementing Nessler's reagent (lmL) to the bacterial culture after 4 days of incubation (Cappuccino and Sherman 1992). Presence of yellow color indicates production of ammonia.

Molecular characterization of bacterial isolates

Genomic DNA of 2 bacterial isolates (A2 and CP19) was extracted according to the method (Bazzicalupo and Fani 1995) and 16S rDNA region was amplified using universal primer (27F: 5´-AGAGTTTGATC-MTGGCTCAG-3´and 1492R 5´-TACCTTGTTAC-GACTT-3´). Amplification was carried out on thermal cycler PTC-200 thermal cycler (MJ Research) and amplified genomic products of both the isolates were sequenced by Chromus biotech at Bangalore, India.

***In silico* analysis of sequence data**

16S rDNA sequences of test bacterial isolates were analyzed for homology with known 16S rDNA sequences available in NCBI (National Center for Biotechnology Information) database using BLAST (Basic Local Alignment Search Tool) (Singh et al. 2012). Sequences were aligned by multiple sequence alignment using Clustal W algorithm program. To deduce the evolutionary relatedness of the aligned sequences, dendrogram was constructed using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) with MEGA 5.0 (molecular evolutionary genetic analysis) software (Tamura et al. 2013). Neighbour joining method generates phylogenetic

tree on the basis of distance matrix calculated from sequence data.

Consortium preparation

Based on preliminary PGPR screening,active bacterial consortia were prepared and designated as Cl. Bacterial consortia were made on the basis of prior testing of compatibility. Initially, a single colony from each bacterial strain was inoculated in Nutrient Broth and incubated at optimum pH and temperature for overnight with continuous shaking (120 rpm). Absorbance was recorded at 600 nm wavelength [OD600] by using UV-Vis Spectrophotometer (Perkin Elmer, Lambda 35). Further, equivalent amount of 100 µL of active culture (A_{600}-0.6) from each strain was transferred to 100 mL of nutrient broth and mixed to prepare the individual consortium. The developed consortium was further evaluated for their plant growth promoting properties (siderophore production, phosphate solubilization and zinc solubilization) and finally selected to prepare carrier based bioformulations.

Carrier based bioformulation

A single colony from each culture was inoculated in 10 mL Nutrient Broth of pH 7 and incubated overnight with continuous shaking (120 rpm) at optimum temperature. Liquid cultures of each bacterium (100 µL) were transferred to 300 mL nutrient broth to scale up and constitute active consortium; 300 mL of prepared active consortium was centrifuged at 10,000 rpm for 10 min. Then, bacterial pellets were incorporated with 10 g sterilized talc under aseptic conditions. The mixture was vortexed for 45 minutes in support of homogenous mixing of talc and bacterial cells and dried at room temperature (28 ± 1^0C). Similar procedure was used to prepare other carrier base bioformulation by using 20 g of autoclaved sugarcane bagasse. After complete drying, talc and sugarcane bagasse based formulations were packed in clean air tight sterilized packets and sealed separately. The experiment was performed in triplicate. The prepared bioformulations were stored at room temperature, in a refrigerator at 4^0C and outdoor under shade conditions.

Shelf life of bioformulation

The vivacity of bacterial cultures in the formulation was confirmed by serial dilution plating method. For this purpose, 25 mg of talc-based formulation was dissolved in 1 mL of sterile distilled water in an eppendorf tube. Later, the suspension was dissolved in 9 mL of sterilized distilled water. The dilution plating was done in nutrient agar medium. The plates were incubated at 28 ± 2 ^{0}C and the viability was evaluated initially on 2nd and 4th day. Therefore, the CFU/mL counts were determined after regular interval of 7 days for subsequent 3 weeks followed by 15-days interval up to 70th day. The above pattern was followed keeping in view, the rapidity of changes in viable counts during storage. The plate counts were carried out in triplicate and the final CFU/mL was the average of three readings.

Statistical analysis

Analysis of variance (ANOVA) was done with statistical software SPSS statistics (version 19.0). All the experiments were done in triplicate and the data shown as mean values ±standard deviation (SD). Results are considered statistically significant at 95% confidence interval ($p < 0.05$).

RESULTS AND DISCUSSION

Zinc solubilization

Zinc solubilizing rhizobacteria are potential substitutes for providing accessible zinc to plants. These microbes convert applied inorganic zinc to their available forms via producing different organic acids or by reducing the rhizospheric pH (Singh et al. 2017, Upadhayay et al. 2018, Khan et al. 2019). Out of 20 bacterial isolates, 2 isolates did not show zone of clearance on basal medium supplemented with ZnO (Table 1). Maximum zinc solubilization was achieved by A9 and CP19 bacterial isolates (Fig. 1).

Phosphate solubilization

The application of proficient phosphate-solubilizing rhizobacteria opens up a new prospect for better crop

Table 1. Plant growth promoting characteristics of bacterial isolates.*, +++ = Highest efficiency, ++ = Moderate efficiency, + = Low efficiency, – = No efficiency.

Sl. No.	Bacterial strains	Siderophore production	ZnO solubilization	P solubilization	IAA production	NH_3 production
1.	A1	-	++	++	+	-
2.	A2	+++	++	+++	+	++
3.	A4	+	++	-	+++	-
4.	A5	++	+	-	-	-
5.	A6	-	++	-	-	-
6.	A7	-	++	++	-	-
7.	A8	+	-	-	-	-
8.	A9	-	+++	++	+	-
9.	A10	-	++		-	-
10.	A12	-	++	-	-	+
11.	A20	-	+	-	++	-
12.	A21	-	++	++	-	-
13.	A22	++	+	+	+++	-
14.	CP1	++	++	-	+	+
15.	CP5	++	-	-	-	+
16.	CP8	++	+	-	+	-
17.	CP10	++	++	++	+	-
18.	CP14	++	+	-	-	+
19.	CP18	+	++	-	-	-
20.	CP19	+++	+++	+	+	-

productivity as well as maintaining soil health (Singh et al. 2011). Under *in vitro* conditions, 8 bacterial isolates solubilized phosphate on Pikovaskaya medium. Bacterium A2 showed highest P solubilization (Fig. 1, Table 1). The major P solubilization mechanisms utilized by soil microorganisms include: Liberation of complexing or mineral dissolving compounds e.g. organic acid anions, release of P during substrate degradation and secretion of extracellular enzymes like acid phosphatase (Khan et al. 2019).

Siderophore production

Iron is highly insoluble in soil and often acts as a limiting factor in the rhizosphere. Some bacteria promote plant growth by producing low density iron chelator biomolecules called siderophore which act as chelating agents for iron (Singh and Singh 2017). In the present study, bacterial isolate A2 showed maximum siderophore production followed by bacterium CP19 (Fig.1). However, 10 isolates showed varying level of siderophore production and 8 bacterial isolates not able to produce siderophore on CAS medium (Table 1).

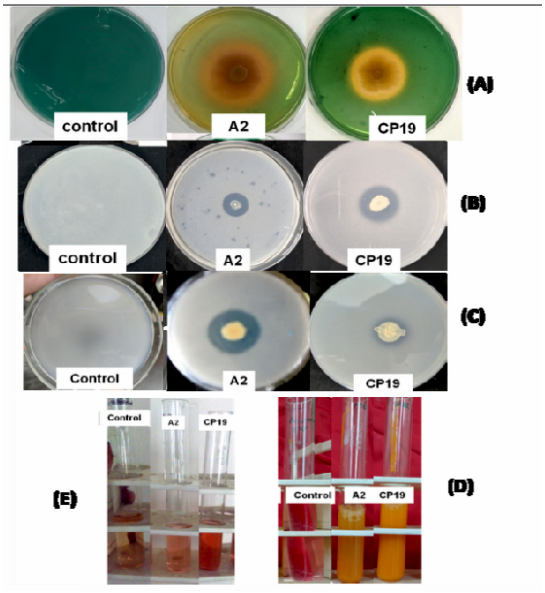

Fig. 1. Plant growth promoting traits produced by bacterial isolates A2 and CP19 (A) Siderophore production, (B) ZnO solubilization, (C) P solubilization, (D) Ammonia production, (E) IAA production.

IAA production

Indole acetic acid (IAA) is one of the most physiologically active auxin. IAA positively influences growth and development of root and shoot, thereby increasing nutrient uptake (Vandana et al. 2018). In the present study, isolate A4 and A22 showed maximum IAA production (Table 1, Fig. 1). Moreover, 8 bacterial cultures showed moderate level of IAA production (Table 1).

Ammonia production

Nitrogen (N) is the most important mineral nutrient required by plants. Ammonia oxidizing bacteria (AOB) are the key operators that are accountable for the conversion of nitrogen into usable forms (Amoo and Babalola 2017). Out of 20, 5 bacterial isolates showed positive results for ammonia production (Table 1). Bacterial isolate A2 gave best results for ammonia production and remaining 4 isolates showed moderate range of ammonia production (Fig. 1).

Molecular characterization

On the basis of best PGP properties, bacterial iso-

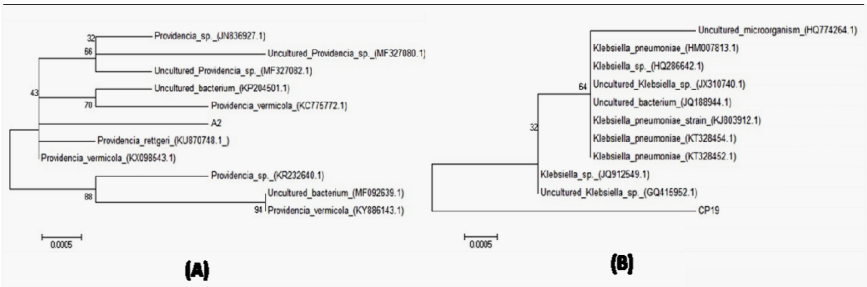

Fig. 2. Phylogenetic tree of isolated bacterial cultures constructed using MEGA 5 software (A) *Providencia vermicola* A2, (B) *Klebsiella pneumoniae* CP19.

lates A2 and CP19 were selected for further studies. Both the isolates were gram negative short rods. For estimation of evolutionary relatedness of selected bacterial isolates along with previously known bacterial species, a neighbour joining method based on partial 16S rDNA sequences was used to construct the phylogenetic tree of the bacterial isolates. Bacterial isolates (A2 and CP19) were identified as *Providencia vermicola* and *Klebsiella pneumoniae* respectively on the basis of BLAST match (Fig. 2).

Shelf life of consortia in carrier based bioformulation

The vitality of the active material in the formulations must be retained to produce its biological effect (Omer 2010). In previous reports, the active entity was found mixed with a number of carrier materials such as water, clay, talc, oil or others to make the formulation (Klayraung et al. 2009). In some formulations, enrichment materials comprising of nutrient-rich medium such as calcium alginate, molasses, trehalose, maltose, glycerol, carboxymethyl cellulose and sucrose are also incorporated (Solanki and Shah 2016). Moreover, the bioformulations are safer to handle, easier to apply, better shelf life for storage and when required,can be applied at the site of action. In the current study, the viability of consortium was maintained in formulation containing 2 economically viable carriers for a period of 70 days. These observations suggested that the consortia were maintained to viable form in developed carrier-based bioformulation. With progression of storage, consortium showed sustained viability and maximum CFU counts of 267 ± 3.53, 260 ± 1.41 and 197 ±4.24 at 4^0C, room temperature and outdoor conditions after 70 days in talc based bioformulation. However, the CFU count of consortium in both carrier based bioformulations was found same under outdoor conditions. Considering the vitality rate of consortium, talc based bioformulation was found to be more effective in contrary to bagasse based bioformulation under 4^0C and room temperature (Table 2). For instance, in bagasse based bioformulation, consortia showed

Table 2. Shelf life of carrier based bioformulation under different storage conditions. *The data are average of triplicate experimental values.

Consortium	Dilution factor	CFU mL^{-1*} at subsequent time intervals (days) 2nd	4th	11th	18th	25th	40th	55th	70th
Talc based bioformulation									
Cl (4^0C)	10^6	280±1.41	276±0.0	272±2.12	270±1.41	268±2.12	269±0.00	268±0.70	267±3.53
Cl (room temperature)	10^7	280±0.70	279±0.70	277±1.41	275±0.70	273±1.41	261±0.70	266±1.41	260±1.41
Cl (outdoor condition)	10^7	276±0.70	270±0.70	266±1.41	243±2.82	214±1.41	209±2.12	197±1.41	197±4.24
Bagasse based bioformulation									
Cl (4^0C)	10^6	201±11.4	197±12.3	192±13.21	187±14.05	186±14.5	185±15.09	185±15.73	168±14.2
Cl (room temperature)	10^6	252±41.01	247±44.54	243±46.6	226±7.07	221±71.41	175±12.09	171±13.50	147±15.68
Cl (outdoor condition)	10^6	276±0.70	270±0.70	266±1.41	243±2.82	214±1.41	209±2.12	197±1.41	197±4.24

significant decrease in cell count number under all conditions with progression of storage after 70 days. These observations recommended that the consortia were found stable and vivacious in talc based bioformulation in contrary to sugarcane bagasse based bioformulation.

CONCLUSION

The present study demonstrates that bacterial co-inoculation can be developed as consortia and used as bioinoculant for eco-friendly agricultural practices. Two bacterial genera based bacterial consortium showed enormously prominent plant growth promoting properties and can be an efficient alternative for chemical fertilizers. Based on the shelf life during 70 days of storage under different conditions, talc based bioformulation proved to be the best supporting carrier material for bacterial growth, survival and biofertilizer preparation. Talc due to its adequate water holding capacity, optimum pH, significant intrinsic moisture content and non-toxic nature, when homogenized with bacterial consortium, displayed good shelf-life and supported utmost population of both the bacterial strain up to 70 days of incubation time. Results of the current study suggest the implication of consortia of plant growth promoting strains may signify a promising way of improving the likelihood due to its PGP properties and enhanced rate of vitality which can be further used as a natural tool to maintain and restore agricultural safety globally. More studies at molecular level and impact on indigenous flora should be made to quickly bring such valuable products in the marketplace. The national bodies in concurrence with the governments at state levels must support the development and research along with entrepreneurship in these forefront areas of plant microbe interaction that can give a support to agro-economy, the back-bone of developing country like India.

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